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Werner Syndrome Protein Is Regulated and Phosphorylated by DNA-dependent Protein Kinase*

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DNA double-strand breaks (DSBs) are a highly mutagenic and potentially lethal damage that occurs in all organisms. Mammalian cells repair DSBs by homologous recombination and non-homologous end joining, the latter requiring DNA-dependent protein kinase (DNA-PK). Werner syndrome is a disorder characterized by genomic instability, aging pathologies and defective WRN, a RecQ-like helicase with exonuclease activity. We show that WRN interacts directly with the catalytic subunit of DNA-PK (DNA-PK$_{CS}$), which inhibits both the helicase and exonuclease activities of WRN. In addition we show that WRN forms a stable complex on DNA with DNA-PK$_{CS}$ and the DNA binding subunit Ku. This assembly reverses WRN enzymatic inhibition. Finally, we show that WRN is phosphorylated *in vitro* by DNA-PK and requires DNA-PK for phosphorylation *in vivo*, and that cells deficient in WRN are mildly sensitive to ionizing radiation. These data suggest that DNA-PK and WRN may function together in DNA metabolism and implicate WRN function in non-homologous end joining.

The rapid recognition and repair of DNA damage is essential for the maintenance of genomic integrity and cellular survival. DNA double-strand breaks (DSBs)[1] are particularly mutagenic when misrepaired and lethal if unrepaired. DSBs are introduced into the genome by several means, including errors in DNA metabolism, ionizing radiation, oxidative damage, and radiomimetic drugs. Many cancer therapies exploit the lethality of DNA DSBs by chemically or physically inflicting this type of damage on cancer cells. Eukaryotic cells have evolved two major pathways to repair DSBs: homologous recombination and nonhomologous end joining (NHEJ). Both pathways contribute significantly to the repair of DSBs and the viability of cells encountering this type of damage (1).

The DNA-dependent protein kinase (DNA-PK) is a key component of the mammalian NHEJ repair pathway. DNA-PK is an abundant nuclear serine/threonine protein kinase consisting of a 460-kDa catalytic subunit, DNA-PK$_{CS}$, and a DNA binding component, Ku. Ku is a heterodimer comprising 69-kDa (Ku70) and 86-kDa (Ku80) subunits (2). The Ku heterodimer binds tightly to DNA DSBs in a sequence-independent manner (3). The kinase function of DNA-PK$_{CS}$ is activated when DNA-PK$_{CS}$ associates with Ku bound to DNA termini. *In vitro*, the heterotrimeric DNA-PK bound to DNA is capable of phosphorylating a wide variety of substrates, but *in vivo* substrates for DNA-PK have yet to be clearly identified (4). Disruption of any of the three genes encoding DNA-PK components, or mutations rendering the kinase inactive, result in severely compromised NHEJ and V(D)J recombination and, in the case of the Ku deficiencies, premature cellular and organismal senescence (5–8). Although DNA-PK and its kinase activity are clearly required for mammalian NHEJ, the specific biochemical function(s) of DNA-PK *in vivo* have yet to be defined.

Werner syndrome (WS) is an autosomal recessive disorder, characterized at the cellular level by genomic instability in the form of variegated translocation mosaicism and extensive deletions (9, 10). Individuals with WS prematurely develop multiple age-related pathologies including bilateral cataracts, graying of the hair, wrinkled skin, osteoporosis, type II diabetes, atherosclerosis, and increased incidence of cancer (11, 12). *WRN*, the gene defective in WS, encodes a 160-kDa protein (WRN), which has 3′-5′ exonuclease, DNA helicase, and DNA-dependent ATPase activities (13, 14). WRN has been reported to interact with p53, replication protein A (RPA), proliferating cell nuclear antigen (PCNA), and DNA polymerase δ, and to associate with the DNA replication complex (15). WRN was also shown to interact with Ku, suggesting a function in DNA repair (16, 17). The enzymatic activities of WRN clearly indicate a function in DNA metabolism, but its specific physiological functions are not yet understood.

Repair of DSBs via NHEJ minimally requires DNA-PK and ligase activity, and frequently a search for microhomology, which may require helicase activity, and exonuclease activity (18). Ligase IV has been identified by genetic and biochemical studies to be the relevant ligase in NHEJ (19), but the enzymes responsible for the exonuclease and helicase activities have not been identified. The Mre11-Rad50-NBS1 complex has been implicated in providing the exonuclease activity involved in the NHEJ, but recent reports suggest that this complex may func-

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[1] The abbreviations used are: DSB, double-strand breaks; NHEJ, nonhomologous end joining; PAGE, polyacrylamide gel electrophoresis; DNA-PK, DNA-dependent protein kinase; EMSA, electrophoretic mobility shift assay; TBE, Tris borate-EDTA; WS, Werner syndrome; M/R/N, Mre11-Rad50-NBS1; IR, ionizing radiation; hTERT, human telomerase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt); DTT, dithiothreitol; bp, base pair(s).

tion primarily in homologous recombination repair, with secondary roles in NHEJ and damage signaling (20, 21). Because WRN has both helicase and exonuclease activities, it could facilitate both the microhomology search and the removal of bases prior to the ligation step of NHEJ.

In this study, we utilize biochemical and cellular approaches to evaluate interactions between DNA-PK and the WRN protein. We show functional and physiological interactions between DNA-PK and WRN. These results provide a mechanistic model for the regulation of WRN activity by DNA-PK. Our results further suggest that WRN and DNA-PK may function together in DNA metabolism.

EXPERIMENTAL PROCEDURES

Cell Lines, and Clonogenic and Viability Assays—WS (73-26) and fibroblasts from a normal sibling (82-6) were infected with a pBABE retrovirus carrying the catalytic component of human telomerase (hTERT) and a puromycin resistance gene, selected, and expanded as described (22). Cultures infected with *insertless* virus senesced 10 (73-26) and 40 (82-6) doublings after infection, whereas cultures infected with pBABE-hTERT continued to proliferate for >150 doublings. Telomerase-expressing cells (73-26 hTERT and 82-6 hTERT) were then superinfected with an LXSN retrovirus carrying the full-length WRN cDNA, untagged or FLAG-tagged at the N terminus, and a neomycin-resistant gene. Infected cells were selected and expanded as described (22). Cells (82-6 hTERT, 73-26 hTERT, and WRN-complemented 73-26 hTERT) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4 mM glutamine, and penicillin/streptomycin. For survival assays, varying numbers of cells (5×10^2 to 2×10^5) were plated in triplicate 100-mm culture dishes and irradiated 4 h later (0–5 Gy) using a Pantak® x-ray generator operating at 320 kV/12 mA. After incubation for 9–15 days, the cells were stained with crystal violet, colonies were counted, and the surviving fraction calculated. For viability assays, cells were plated in triplicate 96-well plates at 1000 cells/well, irradiated as described above, and 9 days later the relative number of viable cells was determined using the CellTiter 96® AQueous assay kit (Promega). This assay is based on cellular conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt) (MTS) to a formazan product that is soluble in culture medium and quantified by absorbance at 490 nm. Absorbance is proportional to the number of living cells.

Protein Expression and Purification—WRN and Ku proteins were purified to near homogeneity from Sf9 insect cells infected with recombinant baculovirus carrying the respective human genes (14, 23). DNA-PK_{CS} was purified from either human placenta or cultured HeLa cells, as described previously (24, 25).

Immunoprecipitation and Western Blot Analysis—Nuclear extracts were prepared by hypotonic swelling and freeze/thaw lysis of cells in 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT). Nuclei were collected by centrifugation at 8000 × *g* for 5 min, and extracted in 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT for 30 min on ice. All buffers contained aprotinin, leupeptin, and pepstatin A at 1 μg/ml, and phenylmethylsulfonyl fluoride at 1 mM. Extracts were clarified by centrifugation at 16,000 × *g* for 10 min and diluted (1:3) with 20 mM Tris-HCl pH 8.0. Mouse IgG (1 μg) or 1 μl of rabbit serum was added to 750 μl of nuclear extract, or 0.5 μg of each purified protein, in 750 μl of Tris-buffered saline containing 1 μg of a 35-bp double-stranded DNA oligonucleotide with 5-nucleotide single-strand extensions on both 5′ termini, (5′-GGC GCA AAT CAA CAC GTT GAC TAC CGT CTT GAG GCA GAG T) (5′-CCG GGA CTC TGC CTC AAG ACG GTA GTC AAC GTG TTG ATT T) as indicated. Reactions were incubated for 2 h at 4 °C with gentle agitation. 10 μl of Ultralink Protein A/G beads (Pierce) was added to the reactions and incubated for 1 h at 4 °C with gentle agitation. The beads were washed with 0.5 ml of Tris-buffered saline, 0.5% Nonidet P-40, boiled in SDS-sample buffer, and the proteins resolved by 6% SDS-PAGE and analyzed by Western blotting using polyclonal antibodies recognizing WRN (26) or monoclonal antibodies recognizing DNA-PK_{CS} (25-4) from NeoMarkers (Fremont, CA).

Electrophoretic Mobility Shift Assays—Binding reactions were carried out in 20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM DTT, and 10% glycerol using 200 fmol of the 35-bp DNA described above. The oligonucleotide was 5′-end labeled on a single strand with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs). Approximately 50 fmol of each purified protein was incubated with the probe for 10 min at 25 °C and resolved by 4.5% non-denaturing Tris/glycine PAGE at 4 °C, and visualized by autoradiography (27). Protein-DNA complexes were cross-linked by adding glutaraldehyde to reactions (0.0625% final concentration) after incubation for 10 min at 25 °C, and continuing the incubation for 5 min prior to electrophoresis (28). Where noted, polyclonal antibodies recognizing WRN were added after cross-linking and incubated for 1–2 min prior to electrophoresis.

Kinase Assays and in Vivo Labeling—*In vitro* kinase assays were carried out as described (29), using ~0.5 pmol (0.2 pmol for wortmannin experiments) of DNA-PK, 1 pmol of WRN, and 0.75 μg of sheared salmon sperm DNA and 1 μM wortmannin where noted. Reactions were separated by 7.5% SDS-PAGE, visualized, and quantified using PhosphorImager and ImageQuant software from Molecular Dynamics (Sunnyvale, CA). *In vivo* labeling experiments used exponentially growing cells (Jurkat, M059J, M059K, SV40 transformed AT (AT5BIVA) or wild-type (1BR3.3GN2) human skin fibroblasts). The cells were washed with phosphate-free RPMI, supplemented with 10% fetal bovine serum, and incubated for 30 min at 37 °C in the same media. [^{32}P]Orthophosphate was added directly to the medium (0.6 mCi/ml), with wortmannin at 20 μM where indicated. After 30 min of incubation at 37 °C, the cells were harvested and lysed in the presence of phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, and 75 nM microcystin) as described previously (30). The extracts were diluted to a final concentration of 100 mM NaCl, Nonidet P-40 was added (0.5%, final concentration), and WRN was immunoprecipitated using 0.5 μl of rabbit anti-serum recognizing WRN. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by autoradiography and Western blotting.

Helicase and Exonuclease Assays—Helicase reactions were carried out in 50 mM HEPES (pH 7.5), 5 mM DTT, 0.1 mg/ml bovine serum albumin, and 2 mM ATP with ~100 fmol of a 21-nucleotide/43-nucleotide duplex DNA substrate in which the 21-nucleotide strand was 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase as described (14). Reactions were incubated for 10 min at 37 °C with ~100 fmol of WRN in each reaction, and the indicated amounts of Ku and DNA-PK_{CS}. Exonuclease assays were carried out under conditions reported previously (14), with the same probe used for electrophoretic mobility shift assays (EMSAs; described above). Briefly, 200 fmol of probe was incubated for 30 min at 37 °C in 50 mM HEPES (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT with ~20 fmol of WRN. Reaction products were resolved on 16% PAGE-TBE gels containing 8.3 M urea.

RESULTS

WRN Interacts Directly with DNA-PK_{CS}—To investigate the possibility that WRN functions in NHEJ, we initially carried out immunoprecipitations to determine whether WRN associates with DNA-PK. A rabbit polyclonal antibody recognizing WRN was used to immunoprecipitate Jurkat cell nuclear extracts. We found that DNA-PK_{CS} co-precipitated with WRN (Fig. 1*A*, *lane 3*). The reciprocal experiment, using a monoclonal antibody that binds DNA-PK_{CS}, co-precipitated WRN with DNA-PK_{CS} (Fig. 1*A*, *lane 4*). The association of WRN with DNA-PK_{CS} was confirmed using extracts from HeLa and M059K cells and, therefore, was not limited to Jurkat cells (data not shown).

The interaction between WRN and DNA-PK_{CS} in cellular extracts could be mediated by other proteins and/or DNA. We therefore reconstituted this interaction using purified proteins and DNA (Fig. 1*B*). WRN associated directly with DNA-PK_{CS}, independent of Ku, and this association was not influenced by the addition of DNA (Fig. 1*B*, *lanes 1–4*). Additionally, the interaction between DNA-PK_{CS} and WRN was not disrupted by ethidium bromide (concentrations up to 100 μg/ml), further indicating that the interaction is direct (data not shown) (31). Interestingly, a significant and reproducible increase in the amount of WRN associated with DNA-PK_{CS} was observed when all four components (WRN, Ku, DNA-PK_{CS}, and DNA) were present (Fig. 1*B*, compare *lanes 3–5* with *lane 6*). WRN has been reported to interact with Ku, and this interaction was shown to stimulate the WRN exonuclease activity *in vitro* (16, 17). Here, the addition of Ku did not appear to influence the association of WRN and DNA-PK_{CS} in the absence of DNA, indicating that the WRN-Ku interaction did not markedly influence the WRN-DNA-PK_{CS} interaction (Fig. 1*B*, compare

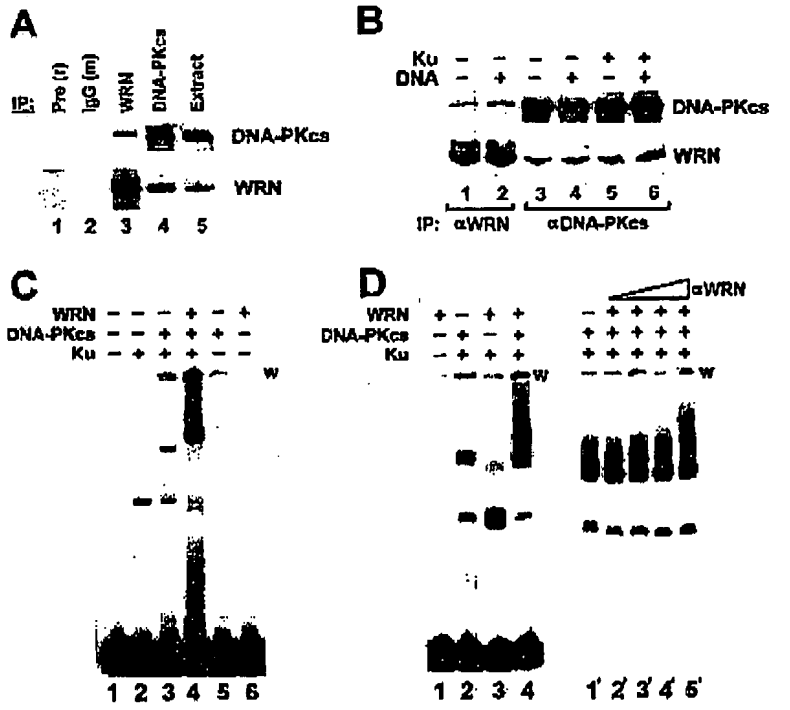

FIG. 1. **Werner protein interacts specifically with the catalytic subunit of DNA-PK and assembles with DNA-PK on DNA.** *A*, Jurkat cell nuclear extract was immunoprecipitated (*IP*) with rabbit pre-immune serum (*lane 1*), normal mouse IgG (*lane 2*), rabbit serum recognizing WRN (*lane 3*), or mouse monoclonal IgG recognizing DNA-PK$_{cs}$ (*lane 4*), or analyzed without immunoprecipitation (extract) (*lane 5*). The extract or immunoprecipitates were probed sequentially for DNA-PK$_{cs}$ and WRN by Western blotting. The protein in the extracts was saturating relative to the amount of antibody. *B*, 0.5 μg of purified of purified WRN, DNA-PK$_{cs}$, and Ku protein was diluted into 750 μl of Tris-buffered saline with or without 1 μg of oligonucleotide DNA as indicated, and immunoprecipitated with anti-WRN or anti-DNA-PK$_{cs}$ antibody. The precipitates were then resolved by SDS-PAGE and immunoblotted for DNA-PK$_{cs}$ and WRN. *C*, EMSA in which 60 fmol of each indicated protein was incubated with 200 fmol of a 5′-^{32}P-labeled 35-bp oligonucleotide and resolved by Tris/glycine electrophoresis under nondenaturing conditions. *D*, EMSA, identical reactions cross-linked with 0.06% glutaraldehyde for 5 min prior to electrophoresis (*lanes 1–5*). Serial dilutions of WRN antibody were added to cross-linked EMSA reactions 1–2 min prior to electrophoresis (2′ = 1:10,000, 3′ = 1:1000, 4′ = 1:100, 5′ = 1:10) and the free probe was run off the gel. The wells on each gel are indicated (*w*).

lane 5 with *lanes 3* and *4*). These data show that WRN interacts directly with DNA-PK$_{cs}$, independently of Ku or DNA, and suggest the formation of a stable WRN·DNA-PK·DNA complex.

WRN Assembles with DNA-PK on DNA—To further characterize the nature of the interaction between DNA-PK and WRN, EMSAs were carried out using purified proteins. The binding of Ku to DNA is well characterized and gave the expected mobility shift (Fig. 1*C*, *lane 2*) (32). The addition of equimolar amounts of purified DNA-PK$_{cs}$ further retarded the mobility of the probe, indicating the assembly of DNA-PK on the DNA (Fig. 1*C*, *lane 3*). The addition of WRN to the DNA-PK binding reaction retarded the probe even further, indicating association of WRN with the DNA-PK·DNA complex (Fig. 1*C*, *lane 4*). The addition of WRN appeared to stabilize the DNA·DNA-PK complex, as evidenced by the distinct increase in the amount of DNA shifted and decrease in the amounts of Ku·DNA and DNA-PK·DNA complexes (Fig. 1*C*, compare *lanes 3* and *4*). Neither DNA-PK$_{cs}$ nor WRN alone retarded the probe under these conditions, indicating that neither protein independently bound DNA efficiently (Fig. 1*C*, *lanes 5* and *6*). Ku plus WRN in the absence of DNA-PK$_{cs}$ gave only the shifted band corresponding to Ku (data not shown).

To detect complexes such as Ku·WRN, which may dissociate under these assay conditions, EMSAs were run after cross-linking with glutaraldehyde (Fig. 1*D*). Cross-linking of WRN in the presence of DNA gave only a faint mobility shift, detectable only after long exposures, indicating that its association with DNA is relatively weak (Fig. 1*D*, *lane 1*). As expected, reactions containing Ku and DNA-PK$_{cs}$ gave two bands corresponding to Ku and assembled DNA-PK (Fig. 1*D*, *lane 2*). Cross-linked reactions containing both Ku and WRN are shown in Fig. 1*D* (*lane 3*). The majority of the shifted DNA had a mobility consistent with the Ku·DNA complex, with only minor bands consistent with WRN·DNA and WRN·Ku·DNA complexes. Finally, the cross-linking of DNA-PK·WRN resolved Ku·DNA, the DNA-PK·DNA complex, and a band with mobility consistent with the DNA-PK·WRN·DNA complex (Fig. 1*D*, *lane 4*). To confirm the presence of WRN in this complex, we carried out a mobility shift experiment using antibodies that recognize WRN. To visualize all three complexes simultaneously, we cross-linked the reactions before adding increasing amounts of WRN antibody (Fig. 1*D*, *lanes 2′–5′*). In the presence of WRN antibody, we observe a mobility shift of only the uppermost band, confirming that WRN is present and exclusive to the uppermost complex. Taken together, these data show that WRN assembles with DNA-PK on DNA and the DNA-PK·WRN·DNA ternary complex is more stable than subcomplexes.

DNA-PK$_{cs}$ Phosphorylates WRN in Vitro and in Vivo—We next asked whether WRN is a substrate for DNA-PK kinase activity. DNA-PK phosphorylated WRN *in vitro* in a DNA-dependent manner (Fig. 2*A*, compare *lanes 3* and *4*). To confirm that the phosphorylation of WRN was due to DNA-PK, we tested whether wortmannin, a compound known to inhibit DNA-PK, could block phosphorylation of WRN. Phosphorylation of WRN was reduced by more than 90% in the presence of 1 μM wortmannin (Fig. 2*A*, *lanes 5* and *6*), confirming that WRN is phosphorylated by DNA-PK and that this phosphorylation is inhibited by wortmannin.

DNA-PK has been shown to phosphorylate a variety of substrates *in vitro*, many of which have no apparent physiological relevance to DNA repair (4). To gain insights into whether WRN is a physiological substrate of DNA-PK, we carried out labeling experiments to determine whether phosphorylation of WRN occurs *in vivo*. Jurkat cells were starved for phosphate, followed by addition of [^{32}P]orthophosphate alone or with the phosphatidylinositol 3-kinase inhibitor wortmannin. Cellular extracts were prepared, followed by immunoprecipitation of WRN. The immunoprecipitates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Sequential autoradiography and Western blotting showed that immunoprecipitated WRN was radiolabeled, clearly demonstrating that WRN was phosphorylated *in vivo* (Fig. 2*B*). Moreover, wort-

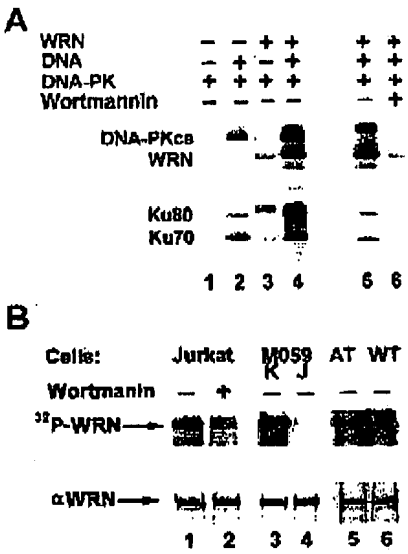

FIG. 2. **Werner protein is phosphorylated by DNA-PK *in vitro* and requires DNA-PK for *in vivo* phosphorylation.** *A*, autoradiogram of an *in vitro* DNA-PK kinase assay, resolved by SDS-PAGE. Reactions contained 0.5 pmol of DNA-PK and 1 pmol of WRN (*lanes 1–4*), or 0.2 pmol of DNA-PK and 1 pmol of WRN (*lanes 5* and *6*), 0.75 μg of sheared salmon sperm DNA, and 1 μM wortmannin where indicated. *B*, Jurkat, M059J, and M059K cells were starved for phosphate for 30 min, then labeled with [^{32}P]orthophosphate prior to extraction and immunoprecipitation. The phosphorylation and protein levels were sequentially analyzed by autoradiography and immunoblotting, respectively.

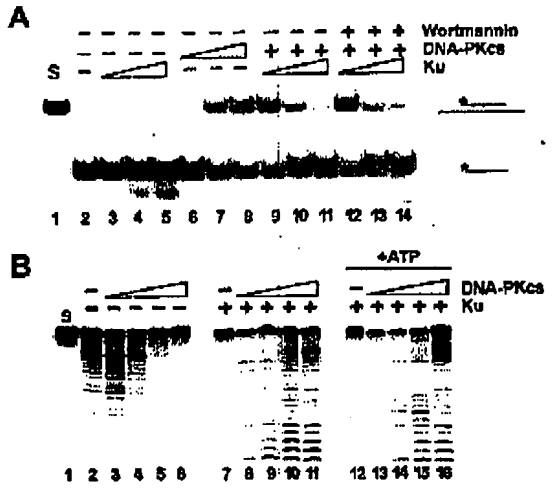

FIG. 3. **DNA-PK$_{CS}$ inhibits WRN exonuclease and helicase activities but inhibition is lost in the presence of Ku.** *A*, WRN helicase assays containing double-stranded DNA substrate 5′-^{32}P-labeled 21-base oligonucleotide annealed to a 43-base oligonucleotide as described (14). *Lane 1* is a mock reaction containing reaction buffer and substrate. *Triangles* indicate increasing amounts of protein (50, 250, and 1000 fmol), and + indicates 1000 fmol of either DNA-PK$_{CS}$ or Ku or 1 μM wortmannin. Reactions were incubated at 37 °C for 10 min with 2 mM ATP. All reactions contained 100 fmol of WRN and were resolved by 10% native TBE-PAGE. *B*, WRN exonuclease assay. All reactions contained 5′-^{32}P-labeled 35-bp oligonucleotide. *Lane 1* is a mock reaction containing reaction buffer and substrate (*S*). *Lanes 2–16* contain 100 fmol of WRN and increasing amounts of DNA-PK$_{CS}$ (0, 50, 250, 500, and 1000 fmol from *left* to *right*) as indicated by *triangles*. *Lanes 7–11* contain 500 fmol of Ku in each reaction, and *lanes 12–16* contain 500 fmol of Ku and 250 μM ATP.

mannin was a potent inhibitor of WRN phosphorylation *in vivo* (Fig. 2*B*, *lanes 1* and *2*).

Wortmannin has been reported to act primarily by inhibiting DNA-PK *in vivo* (33), but significant inhibition of other members of the phosphatidylinositol 3-kinase family of kinases has also been reported (34, 35). To determine whether DNA-PK is required for phosphorylation of WRN *in vivo*, the human glioma cell lines M059J (which lack DNA-PK$_{CS}$) and M059K (which express DNA-PK$_{CS}$) were labeled and analyzed as above (36). WRN immunoprecipitated from ^{32}P-labeled M059K cells was clearly radiolabeled, whereas WRN immunoprecipitated from ^{32}P-labeled M059J was not (Fig. 2*B*, *upper panel*, *lanes 3* and *4*). Western blotting showed that equal amounts of WRN protein were precipitated from M059J and M059K cells (Fig. 2*B*, *bottom panel*, *lanes 3* and *4*). The intensity of minor contaminating proteins in the immunoprecipitates was independent of the presence of wortmannin, or DNA-PK$_{CS}$, indicating that approximately equal amounts of isotope were internalized by the cells, and that other kinase activities were not altered (data not shown). The level of another phosphatidylinositol 3-kinase member, the ataxia-telangiectasia mutated protein, is low in M059J compared with M059K cells (37). To determine whether ataxia-telangiectasia mutated protein levels affected WRN phosphorylation *in vivo*, we immunoprecipitated WRN from ^{32}P-labeled normal (1BR3.3GN2) and ataxia-telangiectasia (AT5BIVA) human fibroblasts. WRN phosphorylation was similar in normal and ataxia-telangiectasia cells (Fig. 2*B*, *lanes 5* and *6*). Thus, the difference in the WRN phosphorylation in M059K and M059J cells was due to the difference in DNA-PK$_{CS}$ levels. These experiments indicate that DNA-PK is required for WRN phosphorylation, and there is a physiological interaction between WRN and DNA-PK *in vivo*.

DNA-PK$_{CS}$ Inhibits WRN Enzymatic Activities and Ku Reverses Inhibition—WRN has both 3′-5′ exonuclease and ATP-dependent helicase activities (13, 14, 38). To determine the functional significance of the interaction between WRN and DNA-PK, we first examined the effect of DNA-PK on helicase activity. Preparations of Ku and DNA-PK$_{CS}$ showed no detectable helicase or exonuclease activity under our assay conditions (data not shown). As reported (16), the addition of Ku did not markedly alter helicase activity (Fig. 3*A*, *lanes 2–5*). However, increasing amounts of DNA-PK$_{CS}$ distinctly inhibited WRN helicase activity (Fig. 3*A*, *lanes 6–8*). This inhibition was relieved by increasing amounts of Ku (Fig. 3*A*, *lanes 9–11*). Because WRN helicase and DNA-PK kinase activities both require ATP, we used wortmannin to specifically inhibit phosphorylation during the helicase reaction. The reversal of helicase inhibition was not altered by wortmannin. Thus, the ability of Ku to reverse the inhibition of WRN helicase by DNA-PK$_{CS}$ was not phosphorylation-dependent (Fig. 3*A*, *lanes 12–14*).

We next investigated the effect of DNA-PK$_{CS}$ on WRN exonuclease activity. Increasing amounts of DNA-PK$_{CS}$ markedly inhibited the exonuclease activity (Fig. 3*B*, *lanes 3–6*). The addition of Ku to a parallel set of reactions alleviated this inhibition (Fig. 3*B*, *lanes 8–11*). WRN exonuclease activity was also stimulated by Ku alone (Fig. 3*B*, compare *lanes 2* and *7*), as reported previously (16). This stimulation is also seen in the helicase assays at high Ku concentrations (Fig. 3*A*, *lanes 4* and *5*; note degradation of the lower band). To determine whether kinase activity alters WRN exonuclease activity, Ku and ATP were added to reactions containing WRN and increasing amounts of DNA-PK$_{CS}$. ATP stimulated exonuclease activity only slightly above that caused by Ku alone, suggesting that reversal of inhibition by Ku is not entirely phosphorylation-dependent (Fig. 3*B*, compare *lanes 8–11* with *lanes 13–16*). Taken

together, these data show that WRN helicase and exonuclease activities are inhibited when WRN is bound to DNA-PK_{CS}, and that this inhibition is reversed when Ku is added.

WS Cells Are Sensitive to Ionizing Radiation (IR) and Wild-type WRN Complements Sensitivity—Together, our data suggest that WRN is regulated by DNA-PK, raising the possibility that WRN functions in NHEJ. If WRN functions in NHEJ, its absence should diminish the cell's capacity for NHEJ, a hallmark of which is sensitivity to IR. WS cells, whether normal or SV-40 transformed have not been reported to be IR-sensitive. However, given the relatively mild symptoms of WS, compared with those in mice deficient in DNA-PK components, WRN may not play an essential role in NHEJ and WRN deficiency may therefore confer only mild sensitivity to IR.

To test this idea, we immortalized fibroblasts from a WS patient (73-26) and normal sibling (82-6) by expressing the catalytic subunit of telomerase (hTERT), as described (22). We then complemented the immortal WS cells with the wild-type *WRN* cDNA, either epitope (FLAG)-tagged or untagged using retroviral transduction and selection of mass populations. Western analysis showed that WRN was undetectable in WS cells, expressed in normal (wild-type) cells, and overexpressed in complemented WS cells (Fig. 4*A*). Clonogenic survival assays showed that WS cells (73-26) were more sensitive to IR than wild-type cells (82-6), although this sensitivity was much less pronounced than in cells deficient in DNA-PK components (8). Most important, WS cells that expressed FLAG-tagged WRN protein showed restoration of wild-type IR sensitivity (Fig. 4*B*). To confirm the IR sensitivity of WS cells, we carried out cell proliferation assays using the MTS assay (39, 40). The results verified the IR sensitivity of WS cells detected by the clonogenic assay. Moreover, they showed complementation of the IR sensitivity by untagged WRN (Fig. 4*C*). In both assays, we used non-clonal populations, thus minimizing the possibility of clonal variation in IR sensitivity, and in the MTS assay, cells infected with an insertless virus were assayed as a control. Although the IR sensitivity of WS cells was mild compared with Ku or DNA-PK_{CS}-deficient cell lines, the sensitivity was complemented by restoration of wild-type WRN protein.

DISCUSSION

Our studies show that WRN associates with DNA-PK_{CS} both *in vivo* and *in vitro*, assembles with DNA-PK on DNA, and forms a stable WRN·DNA-PK·DNA complex. The association of WRN and DNA-PK_{CS} inhibited both the exonuclease and helicase activities of WRN, and the addition of Ku and subsequent formation of the WRN·DNA-PK·DNA complex resulted in active exonuclease, helicase, and kinase activities. Additionally, WRN was phosphorylated *in vivo* in a manner strictly dependent upon DNA-PK_{CS}, and an absence of WRN protein conferred mild IR sensitivity to cells. Taken together, our data suggest that DNA-PK may regulate WRN activity and that WRN functions with DNA-PK to process DNA DSBs.

DNA repair is a metabolic necessity of the highest priority, carried out by a complex network of repair proteins and pathways. The NHEJ repair pathway repairs a significant proportion of DNA DSBs in higher eukaryotes (41). Although exonuclease activity in NHEJ has been clearly demonstrated (42), the lack of exonuclease mutant cell lines exhibiting severe IR sensitivity suggests redundancy for exonuclease function in NHEJ. The Mre11·Rad50·NBS1 (M/R/N) complex has been implicated as functioning directly in NHEJ (43), but other studies indicate DNA-PK and M/R/N do not associate (44) or co-localize (45) and that Ku inhibits M/R/N exonuclease activity (46). Consequently, the role of M/R/N in NHEJ remains unclear. In contrast, Ku has been reported to interact directly with WRN and stimulate WRN exonuclease activity suggesting that these proteins function together in DNA metabolism (16, 17). Our data suggest that WRN functions with DNA-PK to facilitate DNA end processing. It is plausible that both WRN and M/R/N function in NHEJ by providing exonuclease activities that are partially redundant. By negatively modulating the exonuclease activity of WRN, DNA-PK may limit the extent of degradation during NHEJ, thereby preventing extensive deletions and increasing the fidelity of repair. In the absence of WRN, other exonucleases that are less regulated may substitute for this function. Consistent with this idea, mutations at the HPRT locus show more extensive deletions in WS cells compared with wild-type cells (10). Moreover, plasmid-based repair assays show increased mutations with larger deletions in WS cells (47).

A clear sequence homology exists between the helicase domains of WRN, *Escherichia coli* RecQ protein, and the yeast SGS1 protein. The *E. coli* RecQ helicase has been shown to have a role in homologous recombination (48) and the *Saccharomyces cerevisiae* SGS1 functions to suppress homologous recombination (49). Five RecQ-like proteins have been identified in humans as compared with the single RecQ helicase in *E. coli* and *S. cerevisiae*. Among these RecQ-like proteins, the exonuclease function of WRN is unique, suggesting that WRN and possibly the other human RecQ-like proteins may have divergently evolved for specialized functions. Consistent with this idea, the three syndromes associated with mutations in three different human RecQ-like helicases, Werner's syndrome, Bloom's syndrome, and Rothmund-Thomson syndrome, have overlapping but markedly different phenotypes. Additionally, complementation of *sgs1* mutant yeast with BLM restores hydroxyurea resistance and suppresses cell growth in the *top3* background, whereas WRN does not, indicating that these proteins are not functionally equivalent (50). Furthermore, the interactions between WRN and DNA-PKcs are not likely to be mirrored in yeast, as this organism lacks DNA-PKcs. Therefore, it is not clear that WRN, or any of the human RecQ-like helicases, are true functional homologues of the *E. coli* RecQ or yeast SGS1.

Our results show for the first time that the catalytic subunit of DNA-PK interacts directly with WRN, and that Ku does not compete or disrupt the DNA-PK_{CS}·WRN complex. The functional consequence of WRN-DNA-PK_{CS} interaction is surprisingly opposite the effect of the WRN-Ku interaction. DNA-PK_{CS} inhibited WRN exonuclease activity, whereas Ku stimulated the same activity. Furthermore, the interaction between Ku and WRN had little effect on WRN helicase activity, whereas we found that DNA-PK_{CS} dramatically inhibits WRN helicase activity. Most importantly, the addition of Ku relieved the DNA-PK_{CS} inhibition of both WRN enzymatic functions.

In addition to demonstrating a direct interaction between WRN and DNA-PK_{CS}, we also showed the assembly of a WRN·DNA-PK·DNA complex. This complex appeared to be more stable than subcomplexes, including WRN-Ku. The stability of the WRN·DNA-PK·DNA complex suggests that the WRN-Ku and DNA-PK_{CS}-WRN interactions are additive, and not competitive, with respect to WRN. Previous reports clearly showed an interaction between WRN and Ku and a resulting stimulation of WRN exonuclease, but not helicase, activity (16, 17, 51). Given that the DNA-PK_{CS}·WRN complex persisted in the presence of Ku, and that DNA-PK is more abundant than WRN in cells, it is feasible that the majority of WRN is associated with DNA-PK_{CS} *in vivo*. If this is the case, then the interaction between WRN and Ku is most relevant in the context of DNA-PK.

We further report the novel finding that DNA-PK phosphorylates WRN *in vitro* and is required for WRN phosphorylation

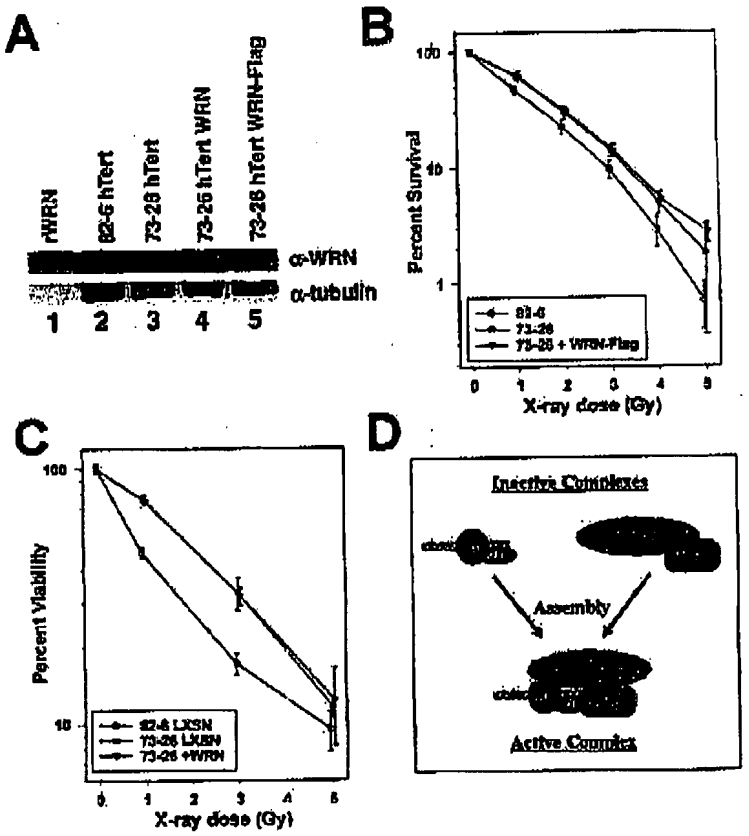

FIG. 4. WS are sensitive to ionizing radiation and sensitivity is reversed by complementation with *WRN* cDNA. *A*, purified recombinant WRN protein (*lane 1*) and equal amounts of the indicated cellular extracts (*lanes 2–5*) were immunoblotted sequentially for WRN and tubulin. *B*, colony formation assay of 82-6 hTERT (*wt*), 76-24 hTERT (*WRN−/−*), and 76-24 hTERT complemented with WRN-FLAG cDNA. Cells were plated in triplicate (500 cells/100-mm dish), irradiated, cultured for 7–12 days, stained with crystal violet, and scored. *C*, MTS viability assay. Cells were plated in triplicate 96-well dishes at 1000 cells/well, irradiated, cultured for 3–9 days, and scored according to the manufacturer's instructions. *Data points* are slightly offset on the *x* axis to avoid overlapping *error bars*. *D*, illustration of DNA-PK-WRN interaction model.

in vivo. These data establish a physiological interaction between DNA-PK and WRN. Although we observed minor effects of phosphorylation on the exonuclease function of WRN, the physiological role of WRN phosphorylation is still unclear. Phosphorylation may function to facilitate the interactions with ligase after end processing is complete, or fulfill an as yet unidentified role in NHEJ or other process. Alternatively, the full effect of WRN phosphorylation may only be apparent when other proteins are present. We showed that WS cells are mildly radiation-sensitive and that this sensitivity was complemented by WRN. Although these data do not support a requirement for WRN in NHEJ, they suggest that WRN may function in a subset of NHEJ events or provide functions that overlap with other proteins such as the M/R/N complex. It is feasible that the type or context of the DSBs dictates the specific exonuclease(s) required to process and rejoin the break. Other enzymes may compensate for the lack of WRN with respect to global NHEJ, whereas the WRN·DNA-PK may be specifically required to process a class of substrates associated with aging. This class of DSBs may involve telomeric DNA, as Ku was recently reported to localize to telomeres in yeast and human cells (52, 53).

Taken together, our data suggest an intriguing mechanism for the regulation of WRN activities by DNA-PK. We show that the WRN·DNA-PK$_{CS}$ complex is inactive with respect to exonuclease, helicase and kinase activities. The WRN·DNA-PK$_{CS}$ complex assembles on DNA termini by associating with the DNA-bound Ku. The assembly of the WRN·DNA-PK·DNA complex then activates the WRN exonuclease and helicase activities, and the DNA-PK$_{CS}$ kinase activity (Fig. 4*D*). We propose that the helicase then functions to unwind the DNA termini, allowing for the microhomology search between the two DNA ends, and the exonuclease functions to remove unpaired bases, allowing ligation. In this model, DNA-PK localizes WRN to DNA breaks, switches on exonuclease and helicase activities, and acts as a scaffold to align DNA termini for microhomology annealing and limited exonucleolytic processing.

Interestingly, this proposed mechanism is consistent with the reported aging phenotypes of Ku and DNA-PK$_{CS}$ knockout mice. Cells from Ku70 and Ku80 knockout mice undergo premature cellular senescence, and the animals are smaller than normal size. Moreover, Ku80 knockout mice prematurely develop multiple characteristics of aging, reminiscent of WS patients (54). DNA-PK$_{CS}$ knockout mice, by contrast, are of normal stature and have not been reported to undergo premature cellular senescence or prematurely develop aging characteristics. Our data shows that WRN is inactive when bound to DNA-PK$_{CS}$, and WRN activation requires association with Ku·DNA. If this were the case in mouse cells, then WRN would be constitutively repressed in the absence of Ku. This would effectively deplete cells for WRN activity and result in a phenotype reminiscent of human WS with respect to aging. In contrast, cells lacking DNA-PK$_{CS}$ cannot repress WRN activity, and WRN-Ku interactions may be sufficient for WRN function, consequently DNA-PK$_{CS}$ knockout mice would not display premature aging phenotypes. Therefore, it is formally possible that the repression of WRN function may be causative in the observed aging phenotype of Ku$^{(-/-)}$ mice.

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